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A homogeneous immunoassay for cyclic nucleotides based on chemiluminescence energy transfer

Anthony K. CAMPBELL and Ashok PATEL
Department of Medical Biochemistry, Welsh National School of Medicine, Heath Park, Cardiff CF4 4XN, U.K.

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1. A chemiluminescent derivative of cyclic AMP, aminobutylethylisoluminol succinyl cyclic AMP (ABEI-scAMP), was synthesized in order to develop a homogeneous immunoassay based on non-radiative energy transfer. 2. ABEI-scAMP was chemiluminescent (5.1×10^{18} luminescent counts·mol^{-1} at pH 13), pure (>95%) stable and immunologically active. 3. A conventional immunoassay was established using ABEI-scAMP and a solid-phase anti-(cyclic AMP) immunoglobulin G which could detect cyclic AMP at least down to 25fmol. 4. A homogeneous immunoassay for cyclic AMP was established by measuring the shift in wavelength from 460 to 525nm which occurred when ABEI-scAMP was bound to fluorescein-labelled anti-(cyclic AMP) immunoglobulin G. The assay was at least as sensitive as the conventional radioimmunoassay using cyclic [^{3}H]AMP and could measure cyclic AMP over the range 1–1000nM. 5. The homogeneous chemiluminescent energy transfer assay was also able to quantify the association and dissociation of antibody–antigen complexes. 6. Chemiluminescence energy transfer occurred between fluorescein-labelled antibodies and several other ABEI-labelled antigens (M_r values 314–150000) including progesterone, cyclic GMP, complement component C9 and immunoglobulin G. 7. The results provide a homogeneous immunoassay capable of measuring free cyclic AMP under conditions likely to exist inside cells.

Since the discovery of cyclic nucleotides (Rall *et al.*, 1957) much has been learnt about the physiological stimuli which alter their tissue content (Robison *et al.*, 1971; Hardman & O'Malley, 1974; Goldberg & Haddox, 1977), about the molecular basis of activation of adenylate cyclase (Ross & Gilman, 1980) and about the phosphorylation mechanisms activated by them (Krebs & Beavo, 1979; Flockhart & Corbin, 1982; Cohen, 1982). In spite of these advances the relative importance of cyclic AMP, cyclic GMP and intracellular Ca^{2+} in the acute and long-term physiological regulation of many cells, as well as in the action of various pathogens, remains confused. It has been proposed that many examples of cell activation and injury, including cell movement, muscle contraction, cell division, secretion and the reversal of the direction of intermediary metabolism, are 'threshold' phenomena (Campbell, 1983). It has further been proposed that these 'thresholds' may be provoked by an appropriate increase in intracellular Ca^{2+} and modified by cyclic nucleotides. One of the crucial experiments required to test this hypothesis is to monitor free Ca^{2+} and free cyclic nucleotide concentrations in intact cells and to correlate changes with the overall response of the cell. The aim of this paper was to establish and characterize a homogeneous assay for cyclic nucleotides which had the necessary properties to be used to determine free cyclic nucleotide concentrations in living cells.

Abbreviations used: ABEI, aminobutylethylisoluminol; ABEI-scAMP (GMP), ABEI succinyl cyclic AMP (GMP); IgG, immunoglobulin.

Chemiluminescence is the emission of light from a chemical reaction and can provide a uniquely sensitive method for measuring substances of biological interest (De Luca, 1978; Campbell & Simpson, 1979; Campbell *et al.*, 1983). Detection of some substances down to 1amol (10^{-18}mol) is possible. The covalent coupling of certain synthetic chemiluminescent compounds, such as luminol, isoluminol and acridinium ester derivatives, to antigens and antibodies has enabled conventional immunoassays to be established of sensitivity comparable with or better than those using radioactive labels (Schroeder *et al.*, 1978; Simpson *et al.*, 1979; Kohen *et al.*, 1980; Kim *et al.*, 1982; Patel *et al.*, 1982). Recently we have reported (Patel *et al.*, 1983) that a homogeneous chemiluminescence immunoassay, not requiring separation of antibody-bound and free antigen, can be established by detecting the shift in wavelength of light emission when a chemiluminescent-labelled antigen is bound to a fluorescent-labelled antibody.

Here we report the application of this principle of non-radiative chemiluminescence energy transfer to the establishment of a homogeneous immunoassay for cyclic nucleotides under conditions compatible with the pH and ionic strength found inside cells.

Experimental

Chemicals and other reagents

Fluorescein isothiocyanate and all organic reagents for the synthesis of ABEI were obtained from Aldrich. $O^{2'}$-Monosuccinyl adenosine 3',5'-monophosphate (succinyl cyclic AMP) was synthesized as described (Steiner *et al.*, 1969; Steiner, 1974) or obtained from Sigma, as were $O^{2'}$-monosuccinyl guanosine 3',5'-monophosphate sodium salt (succinyl cyclic GMP sodium salt), ATP disodium salt, ADP, AMP, GTP, cyclic AMP, cyclic GMP and microperoxidase (MPII). Cyclic [2,8-^{3}H]AMP (36 Ci/mmol) was obtained from Amersham International. Human complement component C9 was purified from human plasma by modification of the method of Biesecker & Müller-Eberhard (1980) (Morgan *et al.*, 1983). Cellulose t.l.c. plates (aluminium backed 20 cm × 20 cm × 0.01 cm) and all other chemicals (analytical grade) were obtained from BDH.

Synthesis of ABEI and ABEI isothiocyanate

ABEI was synthesized from 4-nitrophthalic acid by the method of Schroeder *et al.* (1978). The melting point was 252–257°C and the overall yield was 2% (Schroeder *et al.*, 1978, quote 255–257°C and 2.5% respectively) so that 2 g of pure ABEI is prepared from 100 g of 4-nitrophthalic acid. The yields at each of the six individual steps were similar to those of Schroeder *et al.* (1978).

ABEI isothiocyanate was prepared by suspending 100 mg of ABEI in 15 ml of water/chloroform (2:1, v/v) at 0°C and adding 2 ml of thiophosgene/chloroform (1:19, v/v) (Britzinger *et al.*, 1949). The suspension was mixed for 6 h on ice, and the chloroform layer was removed and dried down under N_2. The residue was redissolved in chloroform, any undissolved material was removed by centrifugation and the chloroform was again removed by evaporation under N_2. The product removed was a single blue fluorescent chemiluminescent spot on silica gel t.l.c. in a solvent system of chloroform/95% ethanol (7:3, v/v). The R_F of ABEI isothiocyanate was 0.75 and that of ABEI was 0.06. The synthetic product had a molecular weight of 318.2 by electron impact mass spectrometry, the theoretical molecular weight of ABEI isothiocyanate being 318.12. No detectable ABEI was found in the purified isothiocyanate preparation either by t.l.c. or by mass spectrometry.

Synthesis of ABEI-scAMP and ABEI-scGMP

These were synthesized using a mixed anhydride coupling reaction (Fig. 1). Succinyl cyclic AMP or succinyl cyclic GMP (20 μmol) were dissolved in 2 ml of dimethyl formamide (dried by refluxing followed by distillation and storage over activated molecular sieve 4A, $\frac{1}{16}$ inch sodium aluminosilicate pellets from Sigma) plus triethylamine (45 μmol) on ice. Isobutyl chloroformate (45 μmol) (Aldrich; stabilized with $CaCO_3$) was added and the solution was incubated for 30–60 min at 0°C. Solid ABEI (45 μmol) was then added and the suspension was incubated overnight at 4°C. Undissolved ABEI was removed by centrifugation. ABEI-scAMP or ABEI-scGMP were purified twice by t.l.c. on cellulose using butan-1-ol/acetic acid/water (12:3:5, by vol.) as solvent. The final material was dissolved off the scraped spot in 1–2 ml of 20 mM-sodium phosphate, pH 7, and ran as a single spot on t.l.c., there being no detectable (<5%) contamination with ABEI or succinyl nucleotide. The solution was stored frozen at −20°C and was stable apparently indefinitely (>6 months).

Synthesis of ABEI-progesterone

This was synthesized by converting progesterone 11α-hemisuccinate (4–6 μmol) to its *N*-hydroxysuccinimide ester and reacting this with ABEI (5 μmol) in 0.13 M-$NaHCO_3$/dimethyl formamide (1:1, v/v) by the method of Kohen *et al.* (1979). ABEI-progesterone was purified twice on silica gel t.l.c. (500 μm) using chloroform/95% ethanol (7:3, v/v) as solvent (R_F values: ABEI-progesterone, 0.61; progesterone, 0.85; ABEI, 0.06).

Coupling of ABEI isothiocyanate to proteins

ABEI isothiocyanate (2.67–1335 nmol), dissolved in dimethyl formamide (100 μl), was added to 1 ml of 0.1 M-Na_2CO_3/$NaHCO_3$, pH 9.5, containing either rabbit IgG (26.7 nmol) or complement component C9 (26.7 nmol). After incubation at 4°C for 12 h the labelled conjugate was purified by dialysis or gel filtration on Sephadex G-50 in 50 mM-sodium phosphate, pH 8.

Coupling of fluorescein to antibodies

A crude IgG fraction was prepared by precipitation with Na_2SO_4 (180 mg/ml final concn.) from sheep anti-(rabbit IgG), rabbit anti-(cyclic AMP), rabbit anti-(cyclic GMP), rabbit anti-C9 and rabbit anti-progesterone sera. IgG (20 mg) in 1 ml of 0.1 M-Na_2CO_3/$NaHCO_3$, pH 9.5, was added to a

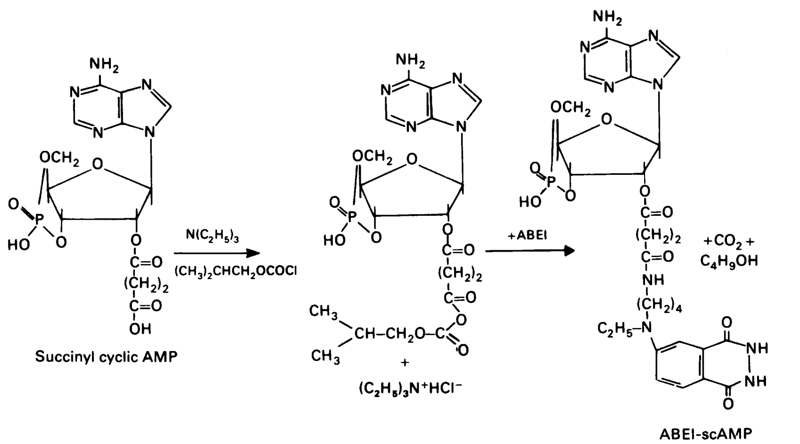

Fig. 1. *Synthesis of ABEI-scAMP*

fresh solution of fluorescein isothiocyanate (10–25-fold molar excess) in the same buffer. After incubation at 4°C for 12h the fluorescein-labelled IgG was purified by dialysis or gel filtration on Sephadex G-50 in 50mM-sodium phosphate, pH 7.4.

Preparation of solid phase anti-(cyclic AMP) IgG

IgG, prepared from rabbit anti-(cyclic AMP) sera, was coupled to dioxocellulose (Hales & Woodhead, 1982). The solid phase anti-(cyclic AMP) IgG was washed and resuspended (2mg of cellulose/ml) in 0.2M-sodium phosphate, pH 7.4, containing 0.02% thiomersal and stored at +4°C.

Radioimmunoassay for cyclic AMP

Radioimmunoassay for cyclic AMP was carried out using rabbit anti-(cyclic AMP) sera and cyclic-[2,8-^{3}H]AMP as label in a total volume of 60 μl as previously described (Siddle *et al.*, 1973).

Assay for ABEI chemiluminescence

A sample (50 μl) of ABEI or ABEI-labelled antigen was added to 100 μl of 5 μM-microperoxidase in either 100mM-sodium barbitone, pH 9, containing 0.01% bovine serum albumin (25 μl of 5M-NaOH was added for assay at pH 13), or 50mM-sodium phosphate, pH 7.4. The sample was placed in front of a photomultiplier tube and 20 μl of 0.175M-H_2O_2 was then added from a spring-loaded syringe to initiate chemiluminescence. Luminescence was recorded as described below over the first 10 or 40s. At pH 9 luminol produced 1.2×10^{19} luminescence counts and ABEI 4.2×10^{18} luminescence counts per mol. Since the background luminescence with H_2O_2 plus microperoxidase alone was 2000 ± 60 luminescence counts in 10s the detection limit for ABEI was approx. 5×10^{-17} mol. Microperoxidase is required for maximum light yield from ABEI (Schroeder & Yeager, 1978; Patel, 1983). It is a hydrolysis product of cytochrome *c* consisting of haem attached to 11 amino acids, with a molecular weight of 1879 (Feder, 1970).

Detection of chemiluminescence

Luminescence was detected digitally by a highly sensitive, low dark current photomultiplier tube (type P4232B from Twentieth Century Electronics, Croydon, Surrey, U.K., or type 9747 AM from EMI Electronics, Ruislip, Middx., U.K.) coupled to an EHT (900–1200V) supply, scalar and discriminator Ecko M5060a as previously described (Campbell & Dormer, 1978), or in a cooled light-tight housing (−20°C) coupled to a specifically constructed digital luminometer with a square wave discriminator, eight-digit scalar and interfaced to an LSI 11 Digital Equipment computer (RT11 operating system) plus dual floppy disc drive.

Detection of chemiluminescence energy transfer

Energy transfer was detected using a specially constructed luminometer containing two photomultiplier tubes, two EHT supplies, two scalars, two transient recorders and two oscilloscopes. Light emission was monitored at two wavelengths simultaneously by placing metal film interference filters type B40 with half band width 7nm (Balzers Ltd., Berkhamsted, Herts., U.K.) in front of each photomultiplier. The apparatus was characterized by comparing the ratio of light emission at 470/510nm from the Ca^{2+}-activated photoprotein obelin, and from the hydroid *Obelia geniculata* (Hallett & Campbell, 1982*a*).

Results

Characterization of ABEI-scAMP

Before establishing a chemiluminescence immunoassay for cyclic AMP it was necessary to demonstrate that the compound synthesized was (i) ABEI-scAMP, (ii) pure, (iii) stable, (iv) chemiluminescent, and (v) immunologically active.

The compound synthesized moved as a single spot on cellulose t.l.c. in a butan-1-ol/acetic acid/water solvent system (Table 1). No ABEI or succinyl cyclic AMP was detectable (<5%). As expected for ABEI-scAMP, the spot was blue fluorescent with an R_F of 0.53, between ABEI (R_F 0.64) and succinyl cyclic AMP (R_F 0.41). After hydrolysis in 0.1 M-KOH for 10 min at room temperature two spots were observed, one corresponding to cyclic AMP (R_F 0.30) and the other a fast-moving blue fluorescent spot presumed to be ABEI-succinate (R_F 0.83). The absorbance spectrum (Fig. 2) was approximately equivalent to the sum of the individual spectra of succinyl cyclic AMP ($\lambda_{max.}$ 258 nm) and ABEI ($\lambda_{max.}$ 290 nm, shoulder between 310 and 320 nm). Since the absorbances due to cyclic AMP at 290 and 315 nm were negligible (ε_{290} 50 and $\varepsilon_{315} < 1$ litre·mol^{-1}·cm^{-1}) the concentration of ABEI in the ABEI-scAMP was estimated from ε_{290} ABEI 18000 and ε_{315} ABEI 12000 litre·mol^{-1}·cm^{-1} (ε_{314} 12500 litre·mol^{-1}·cm^{-1}; Schroeder *et al.*, 1978) and the concentration of cyclic AMP from ε_{258} cyclic AMP 14100 and ε_{258} ABEI 7000 litre·mol^{-1}·cm^{-1}. The result was a ratio of cyclic AMP to ABEI of approx. 1:1, consistent with the compound being ABEI-scAMP.

ABEI-scAMP was stable at room temperature in phosphate buffer, pH 7–8, over a period of several hours and apparently indefinitely (>6 months) at −20°C. It was chemiluminescent, producing 5.1×10^{18} luminescent counts·mol^{-1} at pH 13, 7.2×10^{17} luminescent counts·mol^{-1} at pH 9 and 5×10^{16} luminescent counts·mol^{-1} at pH 7.4 when activated by microperoxidase and H_2O_2 (background at pH 7.4 was 200 luminescence counts in 10 s and at pH 9 was 2000 luminescence counts in 10 s). This compared with the chemiluminescence of ABEI at pH 13 and pH 9 being 6×10^{18} and 2.1×10^{18} luminescence counts·mol^{-1} in 40 s and 10 s respec-

Table 1. *R_F values on t.l.c.*

Aliquots (10 μl) were spotted on to 20 cm × 20 cm × 0.01 cm cellulose aluminium-backed t.l.c. sheets, containing fluor (254 nm). The spots were dried and the chromatogram was developed for 4–6 h in butan-1-ol/acetic acid/water (12:3:5, by vol.) at room temperature (approx. 20°C). Results represent means ± S.E.M. for *n* estimations.

Substance	R_F	*n*
ABEI-succinate?	0.83 ± 0.03	6
ABEI	0.64 ± 0.02	11
ABEI-scAMP	0.53 ± 0.03	7
ABEI-scGMP	0.48	2
Succinyl cyclic AMP	0.41 ± 0.01	9
Cyclic AMP	0.30 ± 0.02	7
Succinyl cyclic GMP	0.28	2
Cyclic GMP	0.16	2
ABEI-scAMP after 0.1 M-KOH for 10 min (two spots)	0.30 and 0.83	2
ABEI-scGMP after 0.1 M-KOH for 10 min (two spots)	0.16 and 0.86	2

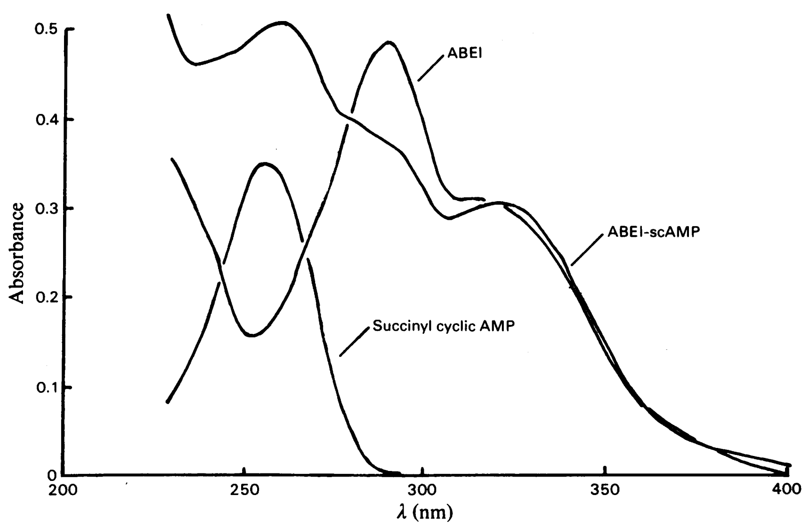

Fig. 2. *Absorbance spectra of ABEI-scAMP, ABEI and succinyl cyclic AMP*

Absorbance spectra were measured in 50 mM-sodium phosphate, pH 7, using a Pye–Unicam SP8.100 spectrophotometer. The concentration of succinyl cyclic AMP and ABEI was 25 μM, equal to that estimated for ABEI-scAMP from A_{314} as described in the Results section.

tively. There was no detectable change in apparent quantum yield or kinetics when ABEI-scAMP was bound to antibody.

Immunological activity of ABEI-scAMP

ABEI-scAMP was shown to be immunologically active by its ability to cross-react with cyclic-[^{3}H]AMP in a conventional radioimmunoassay. The affinity of the rabbit antisera for succinyl cyclic AMP was approx. 10-fold that for cyclic AMP, whereas the affinity for ABEI-scAMP was only 1–2-fold that for cyclic AMP.

By using a solid phase anti-(cyclic AMP) IgG a chemiluminescence immunoassay was established (Fig. 3). Scatchard analysis of the binding of ABEI-scAMP to the solid phase measured by chemiluminescence produced a K_D for ABEI-scAMP of approx. 10^{-9}M. This was carried out in a total volume of 100 μl of 50 mM-sodium phosphate, pH 7.4, containing 60 fmol–2 pmol of ABEI-scAMP, as described in Fig. 3.

Chemiluminescence energy transfer

In order to establish chemiluminescence energy transfer between ABEI-scAMP a dilution curve of fluorescein-labelled anti-(cyclic AMP) IgG, in the presence of a fixed concentration of ABEI-scAMP, was established (Fig. 4). No separation step was used and chemiluminescence was initiated at pH 9, a pH not disturbing antibody–antigen binding, and monitored at two wavelengths (460 and 525 nm) simultaneously. The ratio of luminescence at 460 nm to that at 525 nm decreased from 4.0 to 1.3 over a range of dilutions from 1:500 to 1:5 (Fig. 4). Unlabelled anti-(cyclic AMP) IgG produced no detectable shift of luminescence towards the green, whereas fluorescein-labelled non-immune IgG produced only a small shift at a 1:5 dilution.

Using a fluorescent IgG dilution equivalent to approx. 50% binding of ABEI-scAMP a standard curve for cyclic AMP was established at pH 7.4 and pH 9 (Fig. 5). The assay was homogeneous, there being no separation of antibody-bound and free antigen before initiation of chemiluminescence. Increasing the concentration of cyclic AMP caused an increase in luminescence ratio at 460 nm/525 nm, i.e. a shift towards the blue. The apparent reduction in sensitivity of the assay at pH 7.4 was because the fluorescent anti-(cyclic AMP) IgG and ABEI-scAMP concentration was 10 times that in the pH 9 assay. The assay was sufficiently specific for cyclic AMP, relative to cyclic GMP, ATP, ADP and AMP, to be used for biological samples (Table 2). Cross-reactivities between these nucleotides and

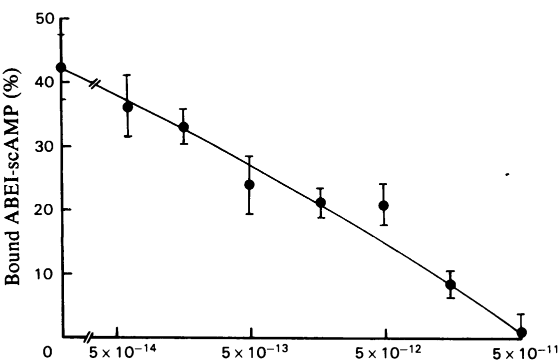

Fig. 3. *Chemiluminescence immunoassay for cyclic AMP*
Cellulose-bound anti-(cyclic AMP) IgG (50 μl; 0.25 mg of cellulose/ml) was incubated with 50 μl of 1 nM-ABEI-scAMP and 50 μl of standard cyclic AMP in 50 mM-sodium phosphate, pH 7.4, for 45 min at 30°C. The tubes were then centrifuged and ABEI chemiluminescence was assayed at pH 9 in 50 μl of supernatant as described in the Experimental section. Results represent means ± S.D. for three determinations.

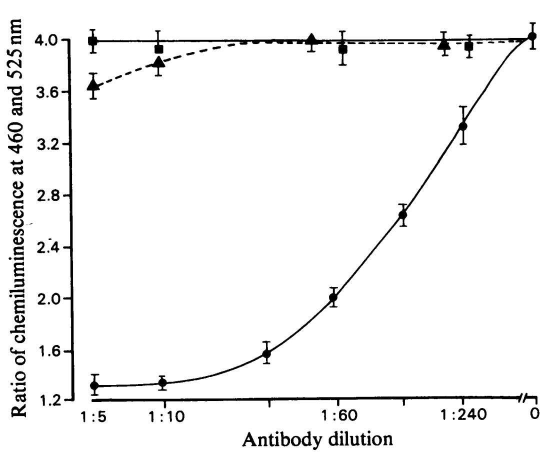

Fig. 4. *Chemiluminescence energy transfer between ABEI-scAMP and fluorescein-labelled anti-(cyclic AMP) IgG*
ABEI-scAMP (50 μl of 10 nM) was incubated with 25 μl of 50 mM-sodium phosphate, pH 7.4, containing 0.1 g of human IgG/100 ml and 25 μl of fluorescein-labelled anti-(cyclic AMP) IgG (●), 25 μl of unlabelled anti-(cyclic AMP) IgG (▲), or 25 μl of fluorescein-labelled non-immune IgG (■), for 2 h at room temperature. The concentration of IgG in all the undiluted solutions was 7 mg/ml; dilutions are as shown. Chemiluminescence was initiated by adding 100 μl of 5 μM-microperoxidase in 100 mM-sodium barbitone, pH 9, containing 10 mg of bovine serum albumin/100 ml, followed by 20 μl of 0.175 M-H_2O_2. Light emission at 460 and 525 nm was measured simultaneously. Results represent the mean ± S.D. for three determinations.

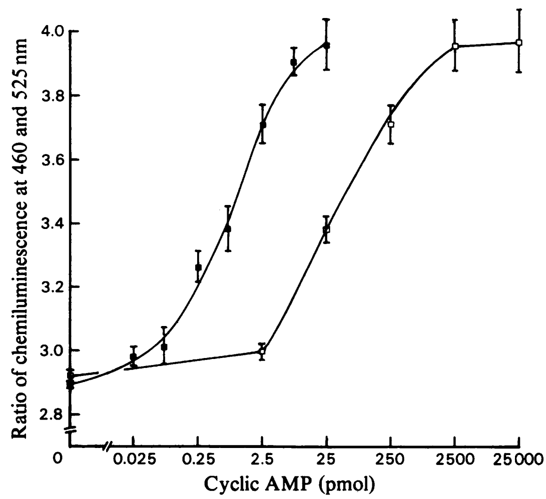

Fig. 5. *Homogeneous immunoassay for cyclic AMP*
Standard cyclic AMP solution (25 μl of 0.1 μM–1 mM) in 50 mM-sodium phosphate, pH 7.4, containing 0.1 g of human IgG/100 ml was incubated with 50 μl of ABEI-scAMP (■, 5 nM; □, 50 nM) and 25 μl of fluorescein-labelled anti-(cyclic AMP) IgG (■, 40 μg of IgG/ml; □, 400 μg of IgG/ml) for 2 h at room temperature. Microperoxidase (100 μl) was then added in 100 mM-sodium barbitone, pH 9 (■), or 50 mM-sodium phosphate, pH 7.4 (□), both containing 10 mg of bovine serum albumin/ml. Chemiluminescence was initiated by adding 20 μl of 0.175 M-H_2O_2 and the ratio of luminescence to background at 460/525 nm was estimated over the first 10 s. Results represent the mean ± S.D. for three determinations.

Table 2. *Cross-reactivity of assay with other nucleotides*
Standard curves for cyclic AMP were established using radioimmunoassay as described in the Experimental section and chemiluminescence energy transfer as described in Fig. 5. Various nucleotides or ABEI were added at a final concentration of 20–100 μM to tubes containing labelled cyclic AMP but no unlabelled cyclic AMP. The binding of label to the antibodies was assayed and the concentration of cyclic AMP equivalent to this binding was calculated. Results are expressed as a ratio of pmol of analyte/pmol of cyclic AMP equivalent and represent the mean of two observations.

Nucleotide	Cross-reactivity relative to cyclic AMP in assay by: Chemiluminescence energy transfer	^3H radioimmunoassay
ATP	4000	6000
ADP	>40000	>40000
AMP	15000	>40000
GTP	5000	4000
Cyclic GMP	>40000	2200
ABEI	—	>1000

cyclic AMP in the heterogeneous cyclic[^{3}H]AMP radioimmunoassay, i.e. requiring a separation step, and the homogeneous chemiluminescence energy transfer immunoassay were very similar (Table 2).

Chemiluminescence energy transfer could also be used to assess the kinetics of antibody–antigen binding, and dissociation on addition of a large excess of cyclic AMP, without needing to separate free and antibody-bound antigen (Fig. 6). This could be carried out over the pH range 7–9 and a temperature range of 0–37°C.

Cyclic AMP estimation in biological samples

In order to demonstrate that the homogeneous chemiluminescence energy transfer assay could be used to measure cyclic AMP in biological samples, cyclic AMP was extracted from pigeon erythrocytes after incubation with adrenaline (10 μM) at 37°C, using the homogeneous immunoassay (Table 3). The results demonstrated not only that the basal and adrenaline-stimulated cyclic AMP content of cell extracts could be determined by energy transfer but also that there was a good correlation between estimations made using energy transfer and those made using the conventional radioimmunoassay ($y = 1.18x + 0.68$; $r = 0.91$; $n = 17$) (Campbell & Siddle, 1976).

Chemiluminescence energy transfer for other antigens

The fact that this phenomenon was not restricted to cyclic AMP was established by studying a range

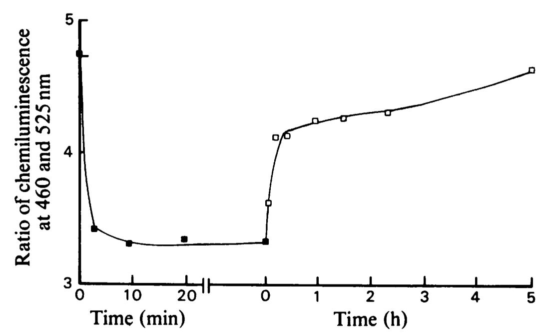

Fig. 6. *Association and dissociation of ABEI-scAMP with fluorescein-labelled anti-(cyclic AMP) IgG*
ABEI-scAMP (1.5 ml of 50 nM) was incubated with 0.75 ml of fluorescein-labelled anti-(cyclic AMP) IgG (100 μg of IgG/ml) in 50 mM-sodium phosphate, pH 7.4, at 37°C. At various times aliquots (75 μl) were removed and chemiluminescence was immediately initiated and measured at 460 and 525 nm at pH 7.4 as described in the legend to Fig. 5. After 1 h 0.4 ml of 1 mM-cyclic AMP was added to 1 ml of the remaining solution at 37°C and the chemiluminescence at 460 and 525 nm was measured in 100 μl aliquots at various times. Results are the mean of two determinations.

of ABEI-labelled haptens and protein antigens (M_r values 314–150000) when bound to fluorescein-labelled IgG, including cyclic GMP, progesterone, complement component C9 and rabbit immunoglobulin (Table 4). In all cases it was possible to establish homogeneous immunoassay standard curves of sensitivity comparable to that of the radioactive counterpart based on a separation immunoassay (Patel, 1983). Apart from cyclic GMP, under the conditions of >95% binding and with the present apparatus (uncorrected for geometry and spectral sensitivity of the photomultiplier tubes), the ratio of luminescence at 460 nm to that at 525 nm shifted from approx. 4, when unlabelled IgG was used, to 1–2 when fluorescein-labelled IgG was used. The reason for the high value of 2.98 with fluorescein-labelled anti-(cyclic GMP) has yet to be established.

Table 3. *Effect of adrenaline on cyclic AMP in pigeon erythrocytes*

Pigeon erythrocytes (5.5×10^8 cells) were incubated in 5 ml of 140 mM-NaCl/5 mM-KCl/2 mM-$MgCl_2$/1 mM-$CaCl_2$/10 mM-Tes (2-{[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]amino}ethanesulphonic acid), pH 7.4, with or without adrenaline (10 μM) at 37°C. At defined time intervals 1 ml samples were taken and 0.2 ml of 1.8 M-$HClO_4$ was added. After centrifugation for 1 min in an Eppendorf microfuge, 1 ml of supernatant was taken and 40 μl of 4 M-K_2CO_3 and 0.4 ml of 0.5 M-potassium phosphate, pH 7, were added. After freeze–thawing, cyclic AMP was estimated by chemiluminescence energy transfer as described in the Experimental section. Results represent means ± S.E.M. of three observations.

Time (min)	Cyclic AMP (pmol/10^6 cells) −Adrenaline	+Adrenaline
0	0.08 ± 0.04	0.09 ± 0.04
5	0.08 ± 0.04	1.72 ± 0.44
15	0.15 ± 0.15	2.93 ± 1.32
30	0.16 ± 0.09	7.17 ± 1.85

Discussion

Non-radiative energy transfer was first predicted by Förster (1948, 1966). It occurs through dipole–dipole resonance between an excited donor molecule and an acceptor molecule, and can be detected by a 'red shift' in the light emitted by the acceptor compared with that from the donor alone. Changes in quantum yield and kinetics have also been observed. This type of fluorescence energy transfer has been applied as a spectroscopic ruler in several biological systems (Stryer & Haugland, 1967; Stryer, 1978). Intramolecular chemiluminescence energy transfer has been observed with phthalazine diones (Roberts & White, 1970; Roswell *et al.*, 1970; Ribi *et al.*, 1972; Gunderman & Roeker, 1976). Intermolecular chemiluminescent energy transfer is apparently rare in luminous organisms, but was first predicted in certain luminous coelenterates including *Aequorea*, *Obelia* and *Renilla* (Johnson *et al.*, 1963; Morin & Hastings, 1971). These organisms contain a green fluorescent protein in addition to the chemiluminescent system. Energy transfer has been demonstrated *in vitro* by a wavelength shift and increased quantum yield with *Renilla* luciferin–luciferase–green fluorescent protein (Ward & Cormier, 1976, 1978).

The results reported here demonstrate a shift in colour of the light emission from a chemiluminescent derivative of cyclic AMP (ABEI-scAMP) from blue (460 nm) to green (525 nm) when it binds to fluorescein-labelled antibodies (Fig. 4). This was interpreted as occurring through energy transfer, as

Table 4. *Chemiluminescence energy transfer with various antigens*

Chemiluminescent labelled antigens were synthesized as described in the Experimental section, and incubated with fluorescein-labelled or unlabelled antibodies (IgG, binding >95% of antigen), or fluorescein-labelled non-immune IgG for 2 h at room temperature (approx. 20°C) in 100 μl of sodium barbitone, pH 9. Chemiluminescence was activated by adding 100 μl of 5 μM-microperoxidase in 100 mM-barbitone buffer, pH 9, containing 0.01 g of bovine serum albumin/100 ml, followed by 20 μl of 175 mM-H_2O_2 in water. Light emission was monitored at 460 nm and 525 nm simultaneously (see the Experimental section) and the ratio was calculated. Results represent the mean ± S.D. of three observations.

Analyte	M_r	Ratio of light at 460 nm/525 nm: Fluorescein-labelled immune IgG	Unlabelled immune IgG	Fluorescein-labelled non-immune IgG
Progesterone	314	1.58 ± 0.15	4.02 ± 0.08	3.92 ± 0.1
Cyclic AMP	329	1.08 ± 0.1	4.01 ± 0.15	3.62 ± 0.12
Cyclic GMP	345	2.98 ± 0.11	3.99 ± 0.06	4.04 ± 0.09
Complement component C9	70000	1.52 ± 0.07	3.94 ± 0.16	3.85 ± 0.2
Rabbit IgG	150000	0.92 ± 0.05	3.96 ± 0.1	3.65 ± 0.08

opposed to quenching, because of the increase in emission at 525 nm concomitant with the decrease at 460 nm. Furthermore this phenomenon was not unique for cyclic AMP but occurred with a wide range of ABEI-labelled antigens (M_r values 314–150000) when these bound to their respective fluorescein-labelled antibodies (Table 4).

On theoretical grounds one would have expected non-radiative energy transfer to occur only when the chemiluminescent labelled antigen was bound to the fluorescent antibody. The efficiency of non-radiative energy transfer is predicted by the Förster (1948) equation:

$$\text{rate of energy transfer} = K_r = d^{-6}\cdot\kappa^2\cdot J\cdot n^{-4}\cdot k_F \times 8.71\times10^{23}\,\mathrm{s}^{-1}$$

$$\text{efficiency of energy transfer} = E = d^{-6}/(r^{-6}+R_0{}^{-6})$$

where d = distance between the centres of the chemiluminescent donor and fluorescent acceptor molecules; κ^2 = the orientation factor for dipole–dipole interactions; J = spectral overlap integral (Fig. 7); n = refractive index of the medium between the donor and the acceptor; k_F = rate constant of fluorescence emission by the donor; $R_0 = (J\cdot\kappa^2\cdot Q_0\cdot n^{-4})^{1/6}\times 97\times10^2\,\mathrm{nm}$, where Q_0 = quantum yield of the chemiluminescent energy donor in the absence of the acceptor. To a first approximation the centres of the donor and acceptor should be within 5–10 nm to give 20–100% efficiency of transfer (Stryer, 1978). The Stokes' radius of IgG (M_r 150000) is about 4 nm. Since antibodies are divalent and the experiments reported here used a range of fluorescent antibodies with a mean of 4–12 fluorescein molecules/molecule of IgG then one would expect most of the ABEI-labelled antigen–fluorescein-labelled antibody complexes to satisfy the conditions for energy transfer. This would not be the case for energy transfer between the chemiluminescence of oxalate esters and fluorescent acceptors. This occurs via electron transfer (McCapra, 1978) only when there is a few tenths of a nm separation between the donor and acceptor. Furthermore, the concentration of either the antigen or antibody would have to be 1–10 mM if non-radiative energy transfer were to occur between the free molecules as opposed to those bound within the antibody–antigen complex. This concentration was some 1000–10000 less than that of ABEI-scAMP or fluorescein-labelled IgG used in the experiments described here (Figs. 4 and 5). The results show that, in spite of the fact that the overlap in the absorption spectrum of fluorescein and the emission spectrum of ABEI (Fig. 7) was not optimal, the J integral was sufficient to allow energy transfer to occur when antibody was bound to antigen.

Chemiluminescent energy transfer has been used to establish a homogeneous assay for cyclic AMP in the pH range 7–9 (Fig. 5), and can measure cyclic AMP down to 25 fmol in a total volume of 100 μl. This is at least comparable with previous radioimmunoassays for cyclic AMP requiring a separation step (Steiner *et al.*, 1969; Siddle *et al.*, 1973; Steiner, 1974). If the reaction were to be carried out in a smaller volume (0.1–1 μl), the chemical blank would be reduced 10^2–10^3-fold and

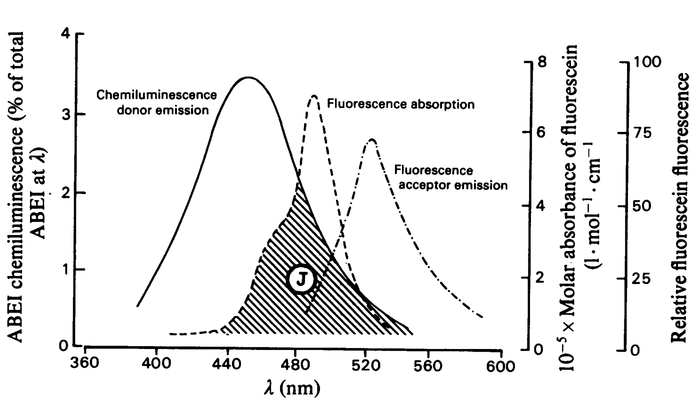

Fig. 7. *J integral for ABEI and fluorescein*

The excitation (– – –) and emission (—·—·) spectra of fluorescein were plotted from the data of Chen (1969), and confirmed by measurement using a Perkin–Elmer 204 A fluorescence spectrometer at pH 7.4 and 9. Chemiluminescence of ABEI (——) was measured at pH 9 as described in the Experimental section in the dual photomultiplier luminometer. Only one photomultiplier had an interference filter in front of it; the other was used as a reference to calculate ABEI light emission as a ratio of light at λ/total light recorded in the reference photomultiplier tube.

amol (10^{-18}mol) quantities of cyclic AMP would then be detectable, sufficient for analysis of single cells (Campbell, 1983). The total cyclic AMP concentration in cells is usually in the range 0.1–10μM. Although free cyclic AMP concentrations have never been measured in cells the homogeneous assay at pH 7.4 (Fig. 5) over the range 10–1000nM should be sufficiently sensitive to do this. The validity of this assay was further established by the good correlation, in cyclic AMP measured in extracts from pigeon erythrocytes (Table 3), between the heterogeneous ^{3}H radioimmunoassay and the homogeneous chemiluminescence energy transfer assay.

The homogeneous assay was also able to assess the rate of association and dissociation of antibody–antigen binding (Fig. 6). By using the luminometer which we have constructed interfaced to the computer (see the Experimental section) it will now be possible to quantify continuously the concentration of antibody–antigen complex at any particular time. The kinetics of association and dissociation of ABEI-scAMP with anti-(cyclic AMP) IgG were fast enough at 37°C to detect changes in free cyclic AMP within a few seconds (Fig. 6). However, in order to avoid any distortion in time course measured in cells by energy transfer it may be necessary to purify a fraction of fast-dissociating antibodies.

These results therefore provide a homogeneous immunoassay capable of measuring cyclic AMP under conditions likely to exist inside cells. The reagents can be entrapped within erythrocyte 'ghosts' and cell hybrids (Hallett & Campbell, 1982*b*; Campbell & Hallett, 1983). It is now necessary to demonstrate whether chemiluminescence can be initiated without damage to the cell, and that the ABEI-scAMP is stable in the cell cytoplasm. If this is the case then this technique will provide a new approach to the study of cell activation, circumventing some of the major limitations of the conventional biochemical analysis on extracts from large numbers of cells.

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